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Analysis of variability in *Cardamom mosaic virus* isolates occurring in India using symptomatology and coat protein gene sequence

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Abstract

Coat protein (CP) gene from six symptomatologically distinct isolates of *Cardamom mosaic virus* (CdMV) causing mosaic disease of cardamom collected from different geographical areas were amplified, cloned, sequenced, compared among themselves and with other CdMV isolates available in the GenBank. Identity of coat protein sequence among the isolates varied from 74.8%–99.3% and 80.9%–99.2% at nucleotide and amino acid levels, respectively. Based on the identity, isolates were classified into three distinct groups. All isolates from Karnataka (except Sirsi) belonged to one group, while isolates from Kerala and Tamil Nadu belonged to the other group. Multiple sequence alignment and phylogenetic analyses confirmed grouping of isolates based on percent identities.

Keywords: cardamom, *Cardamom mosaic virus*, mosaic, RT-PCR, sequence diversity

Mosaic disease caused by *Cardamom mosaic virus* (CdMV) is a major production constraint of cardamom in producing countries (Venugopal & Naidu 1981; Gonsalves *et al.* 1986). In early infected plants, crop loss would be 100% whereas, late infection results in gradual decline in productivity and crop losses to a magnitude of 10%–60%, 26%–91% and 82%–92% were reported in first, second and third years of production, respectively (Varma 1962). The disease is characterized by prominent discontinuous yellowish stripes running out from midrib to the margin of young leaves. In the advanced stages, size of the leaves gets reduced, the plants looses vigour and becomes stunted. The virus is transmitted by the aphid (*Pentalonia nigronervosa* f. *caladii* Van der Goot) in a non-persistent manner (Siddappaji & Reddy 1972; Venugopal 2002). CdMV is a ssRNA virus belonging to the genus, *Macluravirus* of the family Potyviridae (Jacob & Usha 2001). CdMV isolates could be differentiated based on symptoms on cardamom and other allied genera of *Zingiberaceae* and also based on coat protein sequences (Jacob *et al*. 2003). In the present study, we have sequenced coat protein gene of six isolates and compared with other isolates to study the sequence diversity among the isolates. Based on geographical origin, the isolates were classified into three groups.

Surveys conducted during 2008–2009 in major

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cardamom growing regions covering 84 plantations in 44 geographical locations of Karnataka, Kerala and Tamil Nadu of South India, six symptomatologically distinct isolates were collected from different regions. The regions included Sirsi (Uttara Kannada), Sakleshpur (Hassan), Appangala (Kodagu) of Karnataka, Meppadi (Wayanad), Pampadumpara (Idukki) of Kerala and Thadiyankudisai (Dindigul) of Tamil Nadu. These isolates were established under insect proof glass house conditions and were subsequently used for coat protein gene sequence diversity analysis.

Total RNA was isolated following the method described by Siju *et al.* (2007). To amplify the coat protein gene of CdMV, a degenerate forward primer (5′CHCARCADTTTCARATGG 3′) representing last 18 bases on the NIb region and a reverse primer (5′CTCAGCAATTTCAATGG3′) based on 3′ untranslated region (UTR) were designed by multiple alignment of all available sequences from GenBank. RT-PCR mixture contained 20 pmol of each of the forward and reverse primers, 10 U ribonuclease inhibitor (Fermentas, USA), 20 U M-MuLV reverse transcriptase (Fermentas, USA), 1.5 U *Taq* DNA polymerase (Fermentas, USA), 1 X PCR buffer (Genei, Bangalore), 10 mM dithiothreitol (Genei, Bangalore) and 25 µM each of the dNTPs (Genei, Bangalore). RT-PCR mixture (40 µl) was added to the tube containing template RNA (10 µl) resulting in a final reaction volume of 50 µl. Amplification was performed in an automated thermal cycler (Eppendorf master cycler gradient) and the program consisted of one cycle at 42°C for 45 min for cDNA synthesis followed by a 35 cycle reaction profile involving 30 s of denaturation at 94°C, 1 min of annealing at 56°C and 1 min of extension at 72°C and a single cycle of final extension at 72°C for 10 min. The reaction products were analysed on 0.8% agarose gel along with 1Kb DNA ladder. The DNA bands were visualized and photographed using UV transilluminator and gel documentation apparatus (Alpha Innotech Corporation, USA).

The RT-PCR product was eluted from the gel using GenElute Gel Elution kit (Sigma-Aldrich, Bangalore), cloned into pTZ57R/T cloning vector (Fermentas, USA) and transformed into competent *Escherichia coli* strain DH-5α using InsTAclone PCR cloning Kit (Fermentas, USA) following manufacturer's instructions. Recombinant clones were identified by PCR as well as restriction endonuclease digestion and selected clones were sequenced from both ends at the automated DNA sequencing facility available at Chromous Biotech, Bangalore. Multiple sequence alignments were made using Clustal X (1.81). Percent identities were determined using Bioedit program (version 5.0.9). Phylogenetic tree was constructed using Neighborhood algorithm in Clustal X with bootstrap analysis (1000 replicates). The nucleotide and amino acid sequences of CP gene of other CdMV isolates used for comparison were obtained from GenBank (Benson *et al.* 1999). The BLAST programme (Altschul *et al.* 1997) was used to identify related sequences available from the GenBank database.

The results of the survey on the incidence of this disease in different small cardamom growing regions were reported earlier by Biju *et al.* (2010). The survey revealed that varieties, ICRI–3 and IISR Kodagu Suvasini were the major varieties grown in Karnataka state, while Njallani Green Gold was the major cultivar grown in the states of Kerala and Tamil Nadu. The symptoms induced on ICRI–3 and IISR Kodagu Suvasini were similar which included prominent light green/ yellow mosaic patches along the veins, while symptoms on Njallani Green Gold varied with regions. In Karnataka, the symptoms included light green with prominent chlorotic streaks while, prominent light green/yellow mosaic patches along the veins were the symptoms on Njallani Green Gold cultivated in Kerala. The other symptoms like mottling of leaf sheath and pseudostem and shorter and slender tillers were common in all infected plants both in Karnataka and Kerala, irrespective of the varieties cultivated. Based on this, six isolates showing distinct symptoms were collected (Table 1 and Fig.1) and used for coat protein gene sequence studies. Variation in symptoms induced by geographically distinct isolates was reported by earlier workers

Table 1. Symptoms observed on different *Cardamom mosaic virus* isolates used in the present study

Isolate	Region (District, State)	Variety/cultivar	Symptoms on Leaves	Pseudostem/ leaf sheath	Fig
Sirsi	Uttara Kannada, Karnataka	ICRI – 3	Whitish green with discontinuous dark green islands	Mild mosaic mottling	1 a
Sakleshpur	Hassan, Karnataka	ICRI – 3	Prominent yellow mosaic patches along the veins	Mild mosaic mottling	1b
Appangala	Kodagu, Karnataka	Njallani Green Gold	Light green with prominent chlorotic streaks	Severe mosaic mottling	1c
Meppadi	Wayanad, Kerala	Njallani Green Gold	Light green with prominent chlorotic streaks	Severe mosaic mottling	1d
Pampadumpara	Idukki, Kerala	Njallani Green Gold	Continuous dark green stripes along the veins	Mild mosaic mottling	1e
Thadiyankudisai	Dindigul, Tamil Nadu	Njallani Green Gold	Prominent yellow mosaic patches along the veins	Mild mosaic mottling	1f

Fig. 1. Symptomatology of *Cardamom mosaic* virus infecting cardamom in different varieties and regions (a) variety ICRI–3 collected from Sirsi region (b) variety ICRI–3 from Sakleshpur region (c) variety Njallani Green Gold from Appangala region (d) variety Njallani Green Gold from Meppadi region (e) variety Njallani Green Gold from Pampadumpara region (f) variety Njallani Green Gold from Thadiyankudisai region.

(Rao 1977; Naidu *et al*. 1985).

RT-PCR amplified an amplicon of ~1050 bp in all the six isolates while no product was obtained from healthy plants (negative control). The amplified DNA fragment of six isolates were cloned and sequenced. Sequenced region contained 1050 to 1055 bp in different isolates. Of this, coat protein (CP) gene consisted of 816–822 nucleotides, potentially coding for 272–274 amino acid residues in different isolates. Sequences were deposited at the GenBank and their accession numbers are listed in Table 2. The coat protein gene sequences of the isolates were compared among themselves and also with corresponding CP genes from other CdMV isolates available in the GenBank (Table 2). The sequence identity of CP gene among six isolates used in the present study ranged from 75.3%–96.9% and 82.4%–98.1% at nucleotide and amino acid level respectively while, identity with other 11 isolates varied from 74.8%–99.3% and 80.9%–99.2% and with other species of

Table 2. Geographical origin and GenBank accession numbers of CdMV isolates used in this study

Designation	Region (District, State)	Accession No.
APG-1	Appangala (Kodagu, Karnataka)	JN 544081 This study
SKP	Sakleshpur (Hassan, Karnataka)	JN 544082 This study
SRS	Sirsi (Uttara Kannada, Karnataka)	JN 544079 This study
PMP	Pampadumpara (Idukki, Kerala)	JN 544077 This study
MPD	Meppadi (Wayanad, Kerala)	JN 544080 This study
TDS	Thadiyankudisai (Dindigul, Tamil Nadu)	JN 544078 This study
SMR	Somvarpet (Kodagu, Karntaka)	AY 609386
MRD	Margodu (Kodagu, Karnataka)	AY 609385
ANM	Anemahal (Hassan, Karnataka)	AY 833735
LKP	Lakshmipura (Hassan, Karnataka)	AY 823986
YSR	Yeslur (Kodagu, Karnataka)	AF 189125
KTP	Kattappana (Idukki, Kerala)	AJ 312774
VDR	Vandiperiyar (Idukki, Kerala)	AJ 308477
KSR	Kursupara (Idukki, Kerala)	AJ 308476
APG-2	Appangala (Kodagu, Karnataka)	AJ 308472
TLM	Thalathamane (Kodagu, Karnataka)	AJ 308475
MDK	Madikeri (Kodagu, Karnataka)	AJ 308474

Macluravirus was < 62% (data not shown). Among other distinct species of the *Macluravirus*, CdMV was closer to *Alpinia mosaic virus*. The deduced amino acid sequence of CP gene showed high level of identity in the core and C-terminal region, whereas the first 39 amino acids from the N-terminus end showed considerable variations. Differences of 6–49 amino acids were observed in the CP among isolates. A stretch of amino acid residues (WCANNGTSSE) which is highly conserved in the genus *Macluravirus* was also found in all the CdMV isolates (position 122– 131). One amino acid deletion was found in six isolates (MDK, SKP, APG-2, SMR, TLM and LKP) at 38th position and two amino acid deletions were observed at 38th and 39th positions in four isolates (MRD, YSR, APG-1 and ANM).

Majority members in Potyviridae show identity of >82% in the CP region among strains of a distinct species while identity between two distinct species vary from 58%–74% (Adams *et al.* 2005). Based on the above criteria, all 17 CdMV isolates are strains of the same species. However, based on percent identities among strains, they could be classified into three groups (Table 3). All isolates of Karnataka (except Sirsi) belonged to one group with an identity ranging from 90.0% to 99.2% while,

Table 3. Grouping of CdMV isolates based on percent identities in the nucleotide (values shown in brackets) and deduced amino acid of coat protein gene. Designation used for each of the isolates is given in Table 2.

Groups and isolates	Per cent identity among groups		
	Group 1	Group 2	Group 3
Group 1 MRD, YSR, APG-1, ANM, MDK, APG-2, SMR, TLM, LKP, SKP	90.0–99.2 (82.2–99.3)	83.5–86.8 (76.1–77.8)	80.9–86.0 (74.8–77.8)
Group 2 SRS		100	83.5–85.7 (76.1–77.8)
Group 3 KTP, VDR, PMP, MPD, KSR, TDS	87.1–98.9 (82.8–97.4)		

the all isolates of Kerala along with one isolate from Tamil Nadu belonged to another group with identity ranging from 87.1%–98.9% (Table 3).

The results of multiple alignments of 17 CdMV isolates based on amino acid sequences of CP were used to generate a phylogram illustrating their phylogenetic relationship. Scrutiny of isolates revealed that all the six isolates of group III (five isolates from Kerala and one isolate from Tamil Nadu) clustered together (Table 3, Fig.2). On the other hand, isolates from Karnataka showed more divergence. Of the ten isolates belonging to group I from Karnataka, three

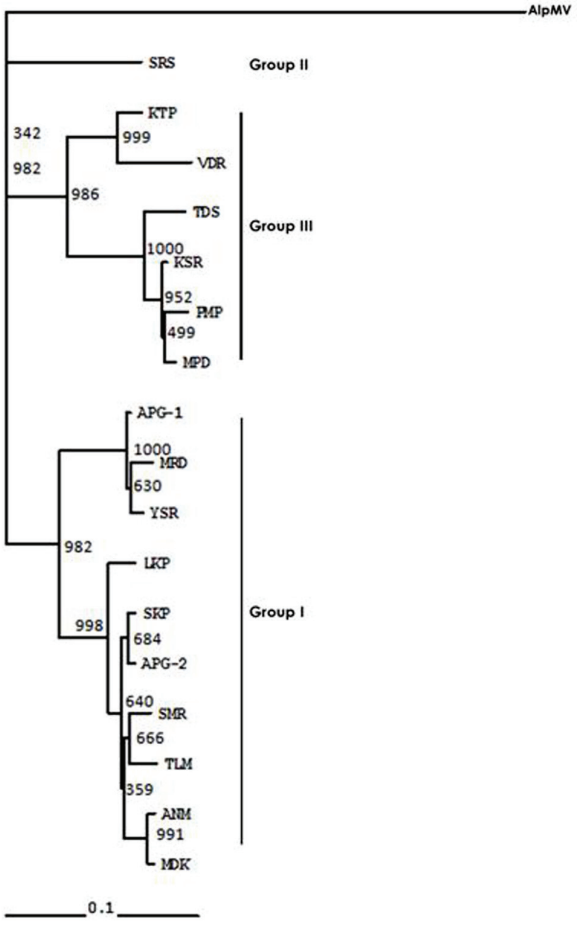

Fig. 2. Phylogram illustrating phylogenetic relationship based on the multiple alignment of the coat protein amino acid sequences of 17 CdMV isolates. *Alpinia mosaic virus* (AlpMV) was taken as outgroup. Details of the isolates provided in Table 2. Bootstrap values are shown at the nodes.

isolates with >98% identities showed close clustering and were slightly away from remaining seven isolates. One isolate belonging to group II (SRS) showed distant relationship with both the other groups (I and III), which clearly indicated its distinctiveness.

The present study on symptoms and sequence analysis of CP gene of CdMV isolates from India revealed existence of high diversity among isolates. Analysis of isolates both based on symptoms and CP gene sequence indicate region wise variation among isolates. All isolates of Kerala more or less showed similar kind of symptoms and showed less variability in the CP region among them. Similarly all isolates of Karnataka (except Sirsi) showed more or less similar symptoms and less variability among CP sequence. Sirsi isolate was quite distinct both in symptoms and CP sequence indicating its distinctiveness. This kind of variation among different potyviruses including CdMV was reported (Bousalem *et al.* 2000; Bateson *et al.* 2002; Jacob *et al.* 2003). Cardamom is mainly grown as a monocrop under the shade trees in slopy forest land both in Karnataka and Kerala, the only exception being at Uttara Kannada (Sirsi) where it is grown as a mixed crop on plain land in arecanut plantations. Region wise variation among isolates observed in the present study probably indicated independent origin and evolution of the virus isolates within each region. RNA virus diversity results from the build up of mutations due to frequent errors in RNA synthesis and recombination events (Roossinck 1997; Simon & Burjarski 1994; Aranda *et al*. 1997).

Existence of high variability in the CP gene among isolates would allow researchers to design strain or species specific primers for the detection of a strain or all strains of CdMV by RT-PCR.

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